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(54) Title: DETECTION OF T CELL STIMULATING TUMOR ANTIGENS

(57) Abstract

The present invention relates to the identification of tumor antigens via analysis of peptides isolated from hapten–modified tumor cells. Both full–length and partial (i.e., peptide) antigens can be identified. The identified antigens form the basis for immunotherapeutic vaccine compositions and methods for treating cancer. According to one embodiment of the invention, major histocompatability complex (MHC) molecules are recovered from hapten–modified tumor cells shown to evoke a specific T cell response. Haptenized peptides are isolated from the MHC molecules and tested for their ability to evoke a specific T cell response. The peptides may then be further analyzed to determine properties such as amino acid sequence by, e.g., mass spectrometry. Methods for using these peptides for identifying new tumor antigens, and for cancer treatment, are described.

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DETECTION OF T CELL STIMULATING TUMOR ANTIGENS

This application claims the benefit of the filing date of Provisional Application Serial No. 60/109,622, filed November 24, 1998, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to the identification of tumor antigens from the structure of peptides from haptenized tumor cells. Both full-length antigens and peptide antigens can be identified. Antigens so identified provide immunotherapeutic vaccine compositions and methods for treating cancer.

BACKGROUND OF THE INVENTION

It was theorized in the 1960's that tumor cells bear tumor specific antigens (TSAs) not present on normal cells, and that the immune response to these antigens might enable an individual to reject a tumor. It was later hypothesized that the immune response to TSA may be increased by introducing new immunological determinants on cells (Mitchison, Transplant. Proc., 1970, 2: 92). Such a "helper determinant", *e.g.*, a hapten, a protein, a viral coat antigen, a transplantation antigen, or a xenogenous cell antigen, could be introduced into a population of tumor cells, and the modified tumor cells injected into an individual with the hope of inducing an immunological reaction against the helper determinant. As a consequence, the immunological reaction against the accompanying TSA would increase, and tumor cells otherwise tolerated would be killed.

Since then, it has been shown that administering hapten-modified melanoma cells to melanoma patients can result in tumor inflammation, tumor

regression and prolongation of patient survival. *See, e.g.*, Berd *et al.*, Cancer Res., 1991, 51:2731-34; U.S. Patent No. 5,290,551 to Berd; Berd *et al.*, Ann. NY Acad. Sci., 1993, 690:147-52; Berd *et al.*, Cancer Immunol. Immunotherapy, 1994, 39:141-147; International Application Ser. No. PCT/US96/09511 published on December 19, 1996 (WO 96/40173).

While the administration of compositions comprising hapten-modified tumor cells has been shown successful for treating cancer, the need remains for additional methods of cancer treatment as well as methods for identifying new tumor antigens.

SUMMARY OF THE INVENTION

Applicants describe herein small molecular weight peptides conjugated to a hapten, having the property of eliciting at least one of the following: (i) a delayed-type hypersensitivity reaction, (ii) tumor infiltration of T lymphocytes, and (iii) tumor inflammation. Furthermore, methods for using the peptides for treatment and identification of new tumor antigens are also described.

The present invention is further directed to a treatment for cancer preferably by administering antigens (peptided or proteins), including hapten-conjugated peptides, identified from tumor cell major histocompatibility (MHC) molecules, or their synthetic or recombinant equivalents. The scope of the present invention includes compositions and methods of treating cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the blocking of T cell response by an anti-MHC class I monoclonal antibody. Expanded CD8-positive (CD8+) T cells were stimulated with autologous dinitrophenyl (DNP) modified B lymphoblastoid cells and the cultures were assayed for gamma interferon after 18 hours. Stimulator cells were preincubated with one of the following: no antibody (none), non-specific mouse IgG (non-specific),

monoclonal antibody W6/32 (class I), or monoclonal antibody L243 (class II).

Figure 2 exhibits MHC restriction of T cell response. Expanded CD8+ T cells (HLA-A1, A2, B8, Bw6) were tested for their ability to proliferate in response to DNP-modified autologous peripheral blood lymphocytes (PBL), and to DNP modified allogeneic PBL, from four other patients. Three of the allogeneic stimulators were matched at one or more class I loci as shown, and the fourth was completely mismatched (designated A24, A26, B44, and B63). Cultures were pulsed with ^{125}IUDR on day 6.

Figures 3A and 3B show the cytotoxicity of DNP-reactive T cells. Melanoma cells, either autologous (auto1) or allogeneic class I-mismatched (allo), were used as targets in a 6-hour ^{51}Cr assay. Effector cells were expanded CD8+, DNP-reactive T cells. The target cells in Figure 3A were haptenized with various concentrations of dinitrobenzene sulfonic acid (DNBS) or trinitrobenzene sulfonic acid (TNBS). The effector: target cell ratio was 20:1. In Figure 3B, target cells haptenized with 2.5 mg/ml DNBS or TNBS were mixed with effector cells at a series of effector: target (E:T) ratios.

Figure 4 shows the T cell stimulatory capacity of the HPLC fractions which were pooled into five groups of ten fractions each. Peptides derived from DNP-modified melanoma cells (DNP-MEL) or DNP-modified B cells (DNP-LY) were stimulatory in pool 2.

DETAILED DESCRIPTION OF THE INVENTION

A method for screening for new tumor antigens by using peptides isolated from MHC molecules of human tumor cells or their chemical, synthetic or recombinant equivalents, is provided. For purposes of the present invention, "a peptide equivalent" is a peptide having the same amino acid sequence as a peptide isolated from an MHC molecule, although prepared using chemical degradation of a protein comprising the peptide, *in vitro* synthesis, or recombinant DNA technology.

For purposes of the present invention, peptides are compounds of two or more amino acids and include proteins. Peptides will preferably be of low molecular weight, about 1,000 kD to about 10,000 kD, or more preferably about 1,000 kD to about 5,000 kD. Preferably, the peptides are isolated from a haptenized tumor cell, and stimulate T cell lymphocytes to produce gamma interferon.

The peptide of the invention may be from about 8 to about 20 amino acids, preferably from about 8 to about 12 amino acids. In addition, the peptide is preferably haptenized. Peptides may be isolated from the cell surface, cell interior, or any combination of the two locations. The extract may be unique to a particular type of cancer cell (versus normal cell). The peptide of the present invention includes, but is not limited to, a peptide which binds to MHC, a cell surface-associated protein, a protein encoded by cancer oncogenes or mutated anti-oncogenes. In one preferred embodiment of the invention, peptides are isolated from MHC molecules, *e.g.*, expressed by the tumor cells or by antigen presenting cells contacted with haptenized tumor cells or tumor cell extracts.

Peptides originally isolated from MHC molecules located on the surface of tumor cells have the property of stimulating T cells. Stimulation for purposes of the present invention refers to proliferation of T cells, as well as production of cytokines by T cells, in response to the cell extract. Proliferation of T cells may be observed by T cell uptake of modified nucleic acids, such as, but not limited to, [^{3}H] thymidine, and ^{125}IUDR (iododeoxyuridine); as well as dyes such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which stains live cells. In addition, production of cytokines such as, but not limited to, gamma interferon (INFγ), tumor necrosis factor (TNF), and interleukin 2 (IL-2) may be tested. Production of cytokines is preferably in an amount of greater than 15 picograms/ml, more preferably about 20 to about 30 picograms/ml, even more preferably about 50 picograms/ml.

Preferably, a tumor cell antigen (peptide or protein) identified in

accordance with the invention is unique, or substantially specific to, a particular type of cancer.

For purposes of the present invention, "modified" includes, but is not limited to, modification with a hapten. Any small molecule that, when administered alone, does not induce an immune response, but enhances immune response against another molecule to which it is conjugated or otherwise attached. may function as a hapten. Generally, the molecule used should have a molecular weight of less than about 1,000 Dalton.

A variety of haptens are known in the art such as, for example: trinitrophenyl (TNP) (Kempkes *et al.*, J. Immunol., 1991, 147:2467); phosphorylcholine (Jang *et al.*, Eur. J. Immunol., 1991, 21:1303); nickel (Pistoor *et al.*, J. Invest. Dermatol., 1995, 105:92); andarsenate (Nalefski and Rao, J. Immunol., 1993, 150:3806).

Generally, the haptens suitable for use in the present invention have the property of binding to a hydrophilic amino acid such as, for example, lysine. A hapten can be conjugated to a cell via the ϵ-amino groups of lysine, or -COOH groups. Additionally, a hapten that can be coupled to hydrophobic amino acids such as tyrosine and histidine *via* diazo functional groups can also be used. Examples of haptens suitable for use in the present invention are: DNP, TNP, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, phosphorylcholine, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard (Nahas and Leskowitz, Cellular Immunol., 1980, 54:241), and combinations thereof. In view of the present disclosure, a skilled artisan would be able to choose haptens suitable for use in the present invention. For example, haptens can be routinely tested using a delayed type hypersensitivity (DTH) test.

Tumor Cells and Components

Tumor cells for use in the present invention may be prepared as

follows. Tumor masses are processed as described by Berd et al., Cancer Res., 1983, 46:2572, which is incorporated herein by reference in its entirety. The cells are extracted by enzymatic dissociation with collagenase and DNAse and/or by mechanical dissociation, frozen in a controlled temperature rate freezer, and stored in liquid nitrogen until needed. On the day that a patient is to be skin tested or treated, the cells are thawed, washed, and may be irradiated to receive a dose of about 2500 cGy. Thereafter, the cells are washed and suspended in Hanks Balanced Salt Solution (HBSS) without phenol red. Conjugation of the prepared cells with DNP is performed by the method of Miller and Claman, *J. Immunol.*, 1976, 117, 1519, incorporated herein by reference in its entirety, which involves a 30 minute incubation of tumor cells with DNFB under sterile conditions, followed by washing with sterile saline.

Cancer cells from a patient may be obtained from a biopsy and frozen until needed. About 100 mg of dinitrofluorobenzene (DNFB) (Sigma Chemical Co., St. Louis, MO) is dissolved in about 0.5 ml of 70% ethanol. About 99.5 ml of PBS is added. The solution is stirred overnight in a 37°C water bath. The cells are thawed and the pellet resuspended in 5 x 10^6 cells/ml in HBSS. 0.1 ml DNFB solution is added to each ml of cells and incubated for about 30 minutes at room temperature. The cells are then washed twice in HBSS.

Cancer cell membranes may be prepared by isolating membranes from a non-modified preparation of cancer cells from a patient. Cells are suspended in about five volumes of about 30 mM sodium bicarbonate buffer with about 1 mM phenyl methyl sulfonyl fluoride and disrupted with a glass homogenizer. Residual intact cells and nuclei are removed by centrifugation at about 1000 g. The membrane fraction is pelleted by centrifugation at 100,000 g for 90 minutes. Following resuspension in about 8% sucrose, the membrane preparation is frozen at about -80° until needed. To a suspension of a membrane preparation (corresponding to about 5,000,000 cell equivalents/ml), about 0.5 ml of 1 mg/ml DNFB is added for about 30

minutes. Similarly, other haptens such as, but not limited to, TNP and N-iodoacetyl-N-(S sulfonic 1-naphtyl) ethylene diamine may be used. Excess DNP is removed by dialyzing the membrane preparation against about 0.15 M PBS for about three days. The membranes are then pelleted.

Alternatively, the cancer cell extract, the peptide, or the membrane preaparation, may be produced by modifying cancer cells from a patient with a hapten such as DNP and then extracting membranes or peptides therefrom.

Haptenized Peptides

From the hapten-modified cells or membranes, peptides may be extracted, some of which are hapten-modified as a result of modifying the cells. Protein extraction techniques, known to those skilled in the art, may be followed by antigen assays to isolate antigen(s) effective for patient treatment. The methods of isolating cell extracts are readily known to those skilled in the art.

In a specific embodiment, cancer cells are isolated from a tumor, cultured *in vitro*, and haptenized in accordance with the method set forth above. Peptides are isolated from the cells according to an established technique of Rotzschke *et al.*, Nature, 1990, 348:252. The cells are treated with a weak acid such as, but not limited to, trifluoroacetic acid. Thereafter, the cell preparation is centrifuged and the supernatant recovered. Compounds having a molecular weight greater than 5,000 are removed from the supernatant by gel filtration (G25 Sepharose, Pharmacia). The remainder of the supernatant is separated on a reversed-phase HPLC column (Superpac Pep S, Pharmacia LKB) using 0.1% trifluoroacetic acid (TFA) and a gradually increasing amount of acetonitrile as eluent (flow rate = 1 ml/min, fraction size = 1 ml). Fractions containing small peptides are obtained by HPLC according to the method of Sambrook et al., *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), concentrated, and may be frozen for later analysis.

As can be readily appreciated by one of ordinary skill, the discovery

that haptenized peptides elicit a tumor-specific immune response suggests an optimized method for isolating such peptides: by hapten-specific affinity chromatography. Haptenized peptides may be isolated from a large number of sources, *e.g.*, the acid extracts from cells as described above, (*see also*, Storkes, *et al.*, J. Immunother., 1993, 14:94) or from affinity-purified MHC molecules (*e.g.*, as described in Hogan *et al.*, Cancer Res., 1998, 58:5144).

Once these peptides are isolated, they can be further purified by affinity chromatography specific for the hapten group. Anti-DNP and anti-TNP antibodies are well known – indeed, these are the classical haptens studies decades ago. Other hapten specific antibodies can be readily generated using ordinary techniques or obtained commercially (*e.g.*, anti-DNP monoclonal antibody SPE-7 from Sigma ImmunoChemicals). Alternatively, a hapten group may confer unique physical-chemical properties, such as hydrophobicity, metal ion binding/chelation, etc., that permit affinity purification.

The HPLC fractions containing small peptides are screened for immunological activity by allowing them to bind to autologous B lymphoblastoid cells which are then tested for ability to stimulate tumor-specific T lymphocytes.

Another method according to the invention involves the use of the T cells elicited in a human patient in response to hapten-modified tumor cells or extracts to screen for tumor-specific antigens.

Peptides are isolated from hapten-modified tumor cells, separated using HPLC, and tested as described in the Examples. T cells used for this testing are isolated from a human patient and propagated *in vitro* as described in International Application No. PCT/US97/15741, published on April 9, 1998 (WO98/14206). The peptides that stimulate T cells are then analyzed for their structure. For example, the peptides are sequenced using methods known in the art. In one embodiment of the invention, the peptides are sequenced as a pool, as described by Burrows *et al.*, J. NeuroSci. Res., 1997, 49:107-116 and Gavin *et al.*, Eur. J. Immunol., 1994, 24:2124-

33, to determine their prevailing motifs. In another embodiment of the invention, the peptides are further separated using methods known in the art, such as HPLC, as described in U.S. Patent Nos. 5,747,269; 5,487,982; 5,827,516 and 5,820,862, and sequenced. Sequencing is performed by using Edman degradation as described in Edman and Berg, *Eur. J. Biochem.*, 80:116-132 (1967) or any modification thereof known in the art.

Preferably, mass spectrometry is employed to determine the structure, and more preferably the sequence, of a haptenized peptide, *e.g.*, as described in PCT Publication WO 98/45700; Das *et al.*, Mol. Biotechnol., 1998, 9:141; Chaurand *et al.*, J. Am. Soc. Mass Spectrom., 1999, 1091. MHC-bound peptides can be directly identified by mass spectrometry (Flad *et al.*, Cancer Res., 1998, 15:5803).

Once a sequence of a peptide isolated from MHC molecules is known, a synthetic peptide having the same sequence may be synthesized and used as a vaccine alone, presented on an antigen presenting cell, and/or in combination with other extracts or whole cells using the methods described above. (*See, e.g.*, PCT Publication Nos. WO 98/33810; WO 97/34613; WO 97/05169; WO 97/04798; WO 95/22561.) Equivalent peptides may also be produced by recombinant techniques or by chemical degradation of proteins containing the isolated peptides.

Peptides may be synthesized on solid phase using F-moc for transient NH_2-terminal protection as described by Atherton *et al., (J. Chem. Soc. Lond. Perkin Trans. 1:* 538, 1981) and characterized by mass spectrometry. All peptides are preferably >90% pure as indicated by analytical HPLC. Lyophilized peptides are dissolved at a concentration of 20 mg/ml in DMSO, diluted to 2 mg/ml in 10 mM acetic acid, and stored at -80°C. Peptides may then be tested in a cytotoxic T lymphocyte (CTL) stimulation assay. The transfectants can also be tested by a chromium release assay.

In another embodiment, the structure of known peptides is altered by changing at least one amino acid, and altered peptides tested for their ability to

stimulate T cells. Assays that can be used are described below and in the Examples.

As the skilled artisan can readily appreciate, once provided with the sequence of a haptenized peptide, it is possible to identify the source antigen. Proteomic databases permit the rapid identification of a protein from which a specific haptenized peptide is derived (*see*, Celis *et al.*, FEBS Lett., 1998, 430:64). In combination with peptide mass fingerprinting (PMS) analysis, the present invention can be used to identify corresponding antgens, including other peptides or proteins, from the haptenized peptide structure (*see, e.g.*, Hogan *et al.*, Cancer Res., 1998, 58:5144).

T Cell Response Assays

Chromium Release Assays - In this peptide sensitization assay, target cells are ^{51}Chromium-labeled for 1 hr at 37°C and washed extensively (Boon *et al.*, *J. Erp. Med.*, 1980, 152:1184-1193). Thereafter, 1000 target cells are incubated in 96-well microplates in the presence of various peptide concentrations for 30 min at 37°C before the addition of CTL cells isolated as described above. Chromium release is measured after incubation for 4 hours at 37°C.

CTL Stimulation Assays - Transfectants can be tested for their ability to stimulate the production of cytokines, such as TNF (Traversari *et al.*, *J. Exp. Med.* 176 1453-1457; Traversari et al. *Immunogenetics*, 1992, 35:145-152); interferon, such as INFγ; interleukins, such as IL-2; and granulocyte macrophage colony stimulating factor (GM-CSF). In the case of TNF, to microliter wells containing target cells, 1500 CTL may be added in 100µl of medium (GIBCO-BRL) containing 10% human serum and 20 U/ml r-human IL-2. After 24 hrs, the supernatant can be collected and the TNF content of the supernatant determined by testing its cytotoxic affect on cells of WEHI-164 clone 13 (Espovik *et al.*, *J. Immunol. Meth.*, 1986, 95:99-105), in a MTT calorimetric assay (Hansen *et al.*, *J.Immunol. Meth.*, 1989, 119:203-210; Travorsari *et al.*, *Inununogenetics*, 1992, 35:145-152).

Tumor Cell Survival Assays - The tumor cells can be labeled with a

fluorescent or radioactive (*e.g.*, ^{51}Cr or ^{125}I-iododeoxyuridine) label. The T cells are added in various numbers. After incubation for about 4 to about 24 hours, the amount of radioisotope released by the tumor cells will be assayed as a measure of tumor cell lysis. Optionally, the retention of fluorescent label may be assayed as a measure of survival of tumor cells. Other assays include simply counting surviving tumor cells before and after incubation with T cells, or counting surviving T cells by their ability to retain a dye such as, but not limited to, MTT.

T Cell Proliferation Assays - The tumor cells are inactivated by irradiation and mixed in varying ratios. After about 1 to about 10 days of incubation, preferably about 5 to about 6 days, a radioisotope that labels T cells DNA is added. Such radioisotopes include, but are not limited to ^{125}-I-iododeoxuridine and ^{3}H-thymidine. The cells are incubated for about 4 to about 18 hours, and the amount of radioactivity incorporated into the cells is measured in a gamma counter.

Treatment

The present invention is directed to cancer immunotherapy. The scope of the invention includes a vaccine composition comprising tumor antigens derived from haptenized peptides isolated from hapten-conjugated human tumor cells, hapten-conjugated tumor cells, and methods of treating cancer by administering the vaccine composition of the invention.

Any malignant tumor may be treated according to the present invention, including both metastatic and primary cancers, as well as solid and non-solid tumors. Solid tumors include carcinomas, and non-solid tumors include hematologic malignancies. Carcinomas include, but are not limited to, adenocarcinomas and epithelial carcinomas. Hematologic malignancies include leukemias, lymphomas, and multiple myelomas. The following are non-limiting examples of the cancers treatable according to the methods of the present invention: ovarian, including advanced ovarian; leukemia, including, but not limited to, acute myelogenous leukemia; and colon, including colon metastasized to liver, rectal,

colorectal, melanoma, breast, lung, kidney, and prostate cancers. The ovarian cancers may be adenocarcinomas or epithelial carcinomas. Colon and prostate cancers may be adenocarcinomas. Leukemias may originate from myeloid bone marrow or lymph nodes. Leukemias may be acute, exhibited by maturation arrest at a primitive stage of development, or chronic, exhibited by excess accrual of mature lymphoid or myeloid cells. Stage I, II, III, or IV cancer may be treated according to the present invention, preferably stages III and IV, even more preferably stage III.

A tumor cell may be a malignant or pre-malignant cell of any type of cancer. In accordance with the present invention, premalignant refers to any abnormal cell which, although not yet a cancer cell, displays certain abnormal changes in genotype or phenotype such as, but not limited to, displastic changes in cervical cells which ultimately may lead to cervical cancer, and displastic nevi which are abnormal skin cells which may lead to melanoma. The tumor cells and extracts preferably originate from the type of cancer to be treated. The tumor cells and extracts may be, but are not limited to, autologous and allogenic cells dissociated from biopsy specimens or tissue culture, as well as stem cells and extracts from these sources. Preferably, the cells and extracts are autologous. Tumor cell extracts of the present invention may be a peptide isolated from a hapten modified cancer cell, or a cell membrane isolated from a hapten modified cancer cell. Alternatively, peptides and membranes may be isolated first and then hapten modified.

The compositions of the invention may be employed in the method of the invention alone or in combination with other compounds, including, but not limited to, other compositions of the invention. Accordingly, cancer cells and cancer cell extracts such as peptides may be used alone or co-administered. For purposes of the present invention, coadministration includes administration together as well as consecutively. In addition, a hapten-modified cancer cell or cell membrane may be co-administered with a hapten-modified peptide or antigen identified therefrom. Further, the compositions may be co-administered with other compounds including

but not limited to cytokines such as IL-2, interleukin-4 (IL-4), INFγ, IL-12, and GM-CSF. The tumor cells and extracts of the invention may also be used in conjunction with other cancer treatments including, but not limited to, chemotherapy, radiation, immunotherapy, and gene therapy.

The compositions of the invention may be administered in a mixture with a pharmaceutically-acceptable carrier, selected with regard to the intended route of administration and according to standard pharmaceutical practice. Dosages may be set with regard to weight, and clinical condition of the patient. The proportional ratio of active ingredient to carrier naturally depend on the chemical nature, solubility, and stability of the compositions, as well as the dosage contemplated. The amounts of tumor cell antigens of the invention to be used depend on such factors as the affinity of the compound for cancerous cells, the amount of antigen and the solubility of the composition. The compositions of the present invention may be administered by any suitable route, including inoculation and injection via, for example, intradermal, intravenous, intraperitoneal, intramuscular, and subcutaneous routes.

In one embodiment the mixture is injected intradermally into 3 contiguous sites per administration on the upper arms or legs, excluding limbs ipsilateral to a lymph node dissection. The vaccine may be reinjected every 4 weeks for a total of about 6 to about 8 treatments. In another embodiment, the vaccine may be injected weekly for at least 4, preferably at least 6 treatments. A booster vaccine may be administered about 6 months after the last vaccination. The drug cyclophosphamide (CY) may be administered 3 days prior to each vaccine administration to augment the immune response to the tumor cells or tumor cell extracts. In one embodiment, CY is only administered prior to the first vaccine. Similarly, a non-haptenized form of the vaccine may be administered.

The compositions of the present invention may be administered alone or in admixture with a pharmaceutical carrier selected with regard to the intended route of administration, and according to standard pharmaceutical practice. Any

known aqueous vehicle useful in drug delivery, such as, but not limited to, saline, may be used in accordance with the present invention as a carrier.

Any adjuvant known to skilled artisans may be useful in the delivery of the present invention. Adjuvants include, but are not limited to, *Bacillus Calmette-Guerin (BCG);* cytokines, such as, but not limited to IL-12, IL-2; and synthetic adjuvants such as, but not limited to, QS-21, (Cambridge Biotech, Worcester, MA) disclosed by Livingston *et al.*, Vaccinc, 1994, 12:1275.

Alternatively, the compositions may be added to antigen presenting cells, which may lead to the presentation of a peptide on their surface. An antigen presenting cell may be, for example, autologous cultured macrophages and autologous cultured dendritic cells. Macrophages are any large ameboid mononuclear cell, regardless of origin, such as, but not limited to, histiocytes and monocytes, which phagocytose, *i.e.,* engulf and digest other cells, dead tissue, degenerated cells, and the like. Macrophages are antigen presenting cells, which present antigens, including tumor antigens, to cells including T cells. Dendritic cells are another example of antigen presenting cells, which appear to be closely related to macrophages, although dendritic cells are more efficient antigen presenting cells than macrophages. Dendritic cells are also potent stimulators of T cells and may be isolated from a variety of body organs and tissues including, but not limited to blood, skin (where dendritic cells are referred to as Langerhans cells), and lymphoid tissues.

The antigen presenting cells that present haptenized peptide or other tumor antigens from a tumor cell on their surface may also be used to immunize patients. Macrophages and/or dendritic cells are extracted from the patient's blood. High concentrations of peptide (about 1 ng/ml to about 1 μg/ml, preferably about 10 ng/ml to about 100 ng/ml), are incubated with the cells overnight or for about 8 hours.

In the treatments methods of the present invention, a method of treating a patient suspected of having cancer, comprises administering a pharmaceutically acceptable amount of a mixture of a tumor antigen (haptenized

peptide, related peptide, or protein) and a composition selected from the group consisting of live tumor cells, tumor cell extracts treated to prevent growth and/or multiplication, and a mixture of tumor cells and tumor cell extracts, including small peptides isolated from MHC molecules or their equivalents. If the composition is a cancer cell extract, the extract may be a peptide or membrane components isolated from a haptenized cancer cell. The composition may be mixed with an immunological adjuvant and/or a pharmaceutically acceptable carrier. Optionally, the administration of a haptenized vaccine may be followed by the administration of a pharmaceutically acceptable amount of a non-haptenized vaccine. A non-haptenized vaccine may also be administered in accordance with the methods of the present invention. Optionally, a pharmaceutically acceptable amount of CY may be administered prior to the vaccine.

The effectiveness of the vaccine may be improved by adding various biological response modifiers. These agents work by directly or indirectly stimulating the immune response. Biological response modifiers of the present invention include, but are not limited to, IL-12 and INFγ. In one embodiment, IL-12 may be administered following each vaccine injection. Administration of IL-12 to patients with inflammatory responses is believed to cause the T lymphocytes within the tumor mass to proliferate and become more active. The increased T cell numbers and functional capacity leads to immunological destruction of the tumors. Dosages for IL-12 will be prepared in accordance with the dosage indications set forth above.

In one embodiment, patients with metastatic cancer may be treated using an immunotherapy regimen with the following components: 1) vaccine consisting of tumor antigen identified according to the invention; and 2) low dose CY pretreatment. Patients are then evaluated to determine whether the tumor has regressed, to monitor tumor inflammatory responses, and to measure DTH to autologous tumor cells, purified protein derivative (PPD), and recall antigens (candida, trichophyton, and mumps). Patients considered to benefit (clinically or

immunologically) from the therapy are continued in the immunotherapy regimen. Subsequent vaccines may be given with or without CY.

The present invention is also aimed at analyzing freshly obtained metastatic tumor biopsies for the presence of cytokine mRNA, which correlates with a productive immune response at the tumor site. For instance, the expression of IFNγ or IL-4 mRNA is characteristic of melanoma metastases that have developed an inflammatory response following administration of DNP-modified autologous vaccine. On the other hand, expression of interleukin-10 (IL-10) mRNA is independent of an inflammatory response and observed in nearly all melanoma biopsy specimens. Examination of cell lines derived from melanoma biopsies as well as in situ PCR analysis has demonstrated that the source of IL10 is the melanoma cells themselves rather than the associated lymphocytes. Similar cytokine analysis is conducted for other tumor metastases.

EXAMPLES

The invention is further illustrated by the following non-limiting examples.

EXAMPLE 1: T Cell Responses

T Cell Proliferation. Circulating T lymphocytes from one of these patients (DM2) (see PCT Publication No. WO 96/40173) were expanded *in vitro* by culture in the presence IL-2 and repeated restimulation with autologous DNP-modified B lymphoblastoid cells. After four weeks of expansion, the T cells were 70% CD3+, CD8+ and 30% CD3+, CD4+. They proliferated when stimulated by autologous DNP-modified B lymphoblastoid cells or DNP-modified cultured melanoma cells, but not by unconjugated autologous cells. These cells were separated by positive panning into CD8-enriched and CD4-enriched populations that were 98% pure as determined by flow cytometry analysis. Both CD4-enriched and CD8-

enriched T cells exhibited a proliferative response to DNP-modified autologous B lymphoblastoid cells. However, only CD8+ T cells responded to DNP-modified autologous melanoma cells.

Expanded T cells were tested for their ability to produce cytokines when stimulated with autologous, DNP-modified B lymphoblastoid cells. They produced INFγ but not IL-4. To determine whether both CD4+ and CD8+ T cells were involved in the cytokine response, sublines obtained by plating T cells at limiting dilution were analyzed. Each of these cultures was homogeneous with respect to expression of CD4 and CD8. Three of these sublines (two CD4+, one CD8+) were tested for cytokine response to DNP-modified B lymphoblastoid cells. All three produced INFγ, while none made IL-4.

Cytokine Production. T cells were added to round bottom microliter plates at 1 X 10^5 cells/well. An equal number of stimulators (DNP-modified autologous B lymphoblastoid cells) was added, and supernatants were collected after 18 hours of incubation. Commercially available ELISA kits were used to measure INFγ (Endogen, Boston, MA; sensitivity = 5 pg/ml) and IL4 (R&D Systems, Minneapolis, MN; sensitivity = 3 pg/ml).

To evaluate the MHC-dependence of the response, stimulator cells were pre-incubated with monoclonal antibodies to MHC class I (W6/32) or MHC class II (L243) at a concentration of 10 μg/ml for one hour before adding responder cells. Non-specific mouse IgG at the same concentration was tested as a negative control.

DNP-reactive CD8+ T cells obtained by panning of the bulk population were capable of long-term (> 3 months) culture in IL-2-containing medium by repeated stimulation with DNP-modified autologous B lymphoblastoid cells while retaining they retained the stable phenotype CD3+, CD8+. Two observations confirmed that their response was MHC class I restricted:

(1) Gamma interferon production was blocked by pre-incubation of

stimulator cells with anticlass I framework antibody, but not by anti-class II antibody (Figure 1). (2) The T cells were able to respond to allogeneic DNP-modified stimulators matched at one or both HLA-A loci, but not to mismatched HLA-A stimulators. As shown in Figure 2, T cells proliferated upon stimulation with DNP-modified autologous PBL (HLA-Al, A2, B8+, Bw6) and with DNP-modified allogeneic PBL that expressed Al or A2 or both; while no response was elicited by DNPmodified allogeneic stimulators that were A1 and A2 negative.

Cytotoxicity. Melanoma targets were labeled for two hours with 51Cr (Amersham Corp, Arlington Heights, IL), and 2500 cells were added to round-bottom microliter wells. Effector cells were then added to achieve a series of E:T ratios. After 6 hours incubation at 37°C, supernatants were removed and counted in a gamma counter. The cytotoxicity of the CD8+ T cell line was tested in a ^{51}Cr-release assay with autologous melanoma cells as targets. To minimize spontaneous ^{51}Cr release, DNP modification was accomplished with DNBS rather than DNFB. T cells lysed DNP-modified autologous melanoma cells but not allogeneic (class I-mismatched) melanoma cells (Figures 3A, 3B). There was a direct relationship between susceptibility to lysis and the degree of DNP modification, as determined by the concentration of DNBS used. Neither autologous nor allogeneic targets modified with TNP were lysed.

EXAMPLE 2: **Dinitrophenyl Modified Tumor Peptides.**

Epstein barr virus (EBV) was added to B lymphoblastoid cells in culture. The B lymphoblastoid cells were transformed into a B cell tumor from the patient's own lymphocytes. Cells from a melanoma metastasis were cultured in RPMI 1640 + 10% fetal calf serum or 10% pooled human serum. Non-adhered cells were removed by washing with RPMI medium. Confluent cell cultures were detached with 0.1% EDTA and passaged into two new flasks for about 10 to about 30 passages. To test for INFγ production by T cells, lymphocytes were collected from a patient's blood

sample. About 1,000,000 lymphocytes were mixed with DNP modified autologous melanoma cells to stimulatc T cells. Every 7 days, 100 U/ml of IL-2 was added. The T cells were expanded by passage as disclosed above. The T cells were then restimulated by the DNP-modified autologous melanoma cells. A population of T cells responsive to the DNP modified autologous melanoma cells was obtained. Stimulatory capacity was determined by the amount of INFγ production by the T cells. Generally, only production of INFγ at greater than 15 picograms/ml was considered, and these T cells were used to test the peptide.

Small peptides were extracted from 4 types of cells, all generated from a single patient: 1) B lymphoblastoid cells, 2) B lymphoblastoid cells modified with dinitrophcnyl (DNP), 3) cultured melanoma cells, and 4) cultured mclanoma cells modified with DNP. Cells wcre suspended in 0.1%. trifluoroacetic acid, dounced, sonicated, and centrifuged at 100,000 xg for 90 minutes. Supernatant components with a molecular weight >10,000 was removed using a Centricon 10 filter. The remaining matcrial was separated on a reversed phase HPLC column. Individual fractions were collected, dried, resuspended in culture medium, and added to autologous B lymphoblastoid cells, which bound and presented the peptides. These peptide-pulsed B cells were tested for ability to stimulate a T lymphocyte cell line specifically sensitized to autologous DNP-modified melanoma cells.

Initially, 50 HPLC fractions (10 μ of each sample) were pooled into five groups of ten fractions each. As shown in Figure 4, only peptides derived from DNP-modified mclanoma cells (DNP-MEL) or DNP-modified B cells (DNP-LY) were stimulatory, and only pool #2 was positive.

Each of the individual fractions of pool #2 were analyzed by performing the T cell stimulation test with each fraction in pool 2; activity was found only in fractions #17 and #18, and DNP-MEL peptide stimulated two-fold more gamma interferon production than DNP-LY.

Thesc results indicate that a single HPLC fraction of low molecular

weight peptide preparation contains the peptide or peptides responsible for stimulation of T cells sensitized to DNP modified melanoma cells.

EXAMPLE 3: Peptide Stimulation Inhibition by Anti-DNP Antibody

This experiment is identical to that described in Example 2 with the follwoing exception: After adding peptide to the B lymphoblastoid cells, just before adding the responding T cells, varying concentrations (1-100 μg/ml) of anti-DNP antibody were added to different samples. The anti-DNP antibody was obtained from ATCC (Hybridoma #CRL-1968). Any stimulation caused by DNP-modified peptides will be inhibited by the antibody.

EXAMPLE 4: Identification of Tumor Related Peptides

Recent advances in technology have enabled the identification of tumor-related peptides associated with MHC molecules which elicit cytotoxic T cell responses, and clinical applications of peptide-based vaccines are now under investigation.

Melanoma cell suspensions obtained from metastatic masses were modified with a hapten, DNP, and administered intradermally with BCG after pretreatment with low-dose CY. This treatment induces inflammatory responses in metastases and also significantly increases disease-free survival and total survival in patients with bulky, resepectable nodal metastases. Immunohistochemical and flow cytometric analyses of postvaccine, inflamed metastases showed marked infiltration of CD8 lymphocytes, and some of the specimens contained mRNA for IFNγ. Novel T cell receptor β chain variable (TCRVß) structures in metastatic melanomas in which inflammation was induced by DNP-modified vaccine have also been observed. Patients receiving DNP-melanoma vaccine developed a strong DTH response to DNP-modified autologous melanoma cells, and, to a lesser extent, to DNP-modified autologous PBLs. This was reflected *in vitro* by proliferation and cytokine production

by PBL obtained from patients after, but not prior to, vaccination. Previous studies of a CD8 T cell line derived from such PBLs indicate that the response to DNP-modified autologous cells was class I MHC restricted. Furthermore, the T cell line responded to allogeneic DNP-modified stimulators matched at one or both HLA loci, but not to HLA mismatched stimulators.

In this example, epitopes recognized by these DNP-specific T cells were characterized. The T cells responded to small DNP-modified peptides associated with the MHC. Unexpectedly, the stimulatory activity appeared to be limited to a single HPLC peptide fraction. These data have been reported (Sata et al., Clin. Immunol. Immunopathol., 1997, 85:265), although the full appreciation of the results is only disclosed in this application.

Materials and Methods

Cells. PBL were obtained from a patient who developed a strong DTH reaction to DNP-modified autologous melanoma cells following DNP vaccine administration. PBL were separated from blood by density gradient centrifugation, suspended in freezing medium, frozen in a control-rate freezer, and stored in liquid nitrogen until use.

A T cell line was established from these PBL by repeated stimulation with DNP-modified autologous melanoma cells (DNP-Mel) as described in Sato et al., Clin. Immunol., 1995, Immunopathol. 74, 35-43. In brief, PBL (2×10^6) were mixed with autologous DNP-modified melanoma cells (4×10^5) in 24-well flat-bottom plates in lymphocyte culture medium (RPMI 1640 supplemented with 10% human AB serum, 2 m*M* L-glutamate, 100 μg/ml 100 U/ml streptomycin/penicillin, 10m*M* Hepes, 1% nonessential amino acids). After 5 days of culture, recombinant IL-2 (a gift of Chiron, Emeryville, CA) was added at 100 U/ml. Expanding T cell cultures were maintained in culture medium with IL-2 and were split as needed to maintain a concentration of approximately 2×10^6 cells in a 22 mm-diameter well. The cultures

were restimulated with DNP-modified autologous tumor cells every 2 weeks. After 4 weeks of incubation, a T cell line specific for DNP-modified autologous melanoma cells was established. The T cell line used for peptide experiments consisted of 51% $CD4^+$ cells and 48% $CD8^-$ cells at the time of study, as determined by flow cytometry. This T cell line responded to DNP-modified autologous melanoma cells by proliferation and IFNγ production. IFNγ production by the T cell line after stimulation with DNP-modified autologous melanoma cells was completely inhibited by an anti-MHC class I antibody but not inhibited by an anti-MHC class II antibody (inhibition of 91 and 12.5%, respectively).

B lymphoblastoid cells were obtained by EBV transfection using standard techniques.

Melanoma cells were enzymatically extracted from metastatic masses from the same patient and cryopreserved as previously described. An autologous melanoma cell line was established from the melanoma cell suspension. This cell line is positive for a melanoma-associated proteoglycan detected by antibody 9.2.27 (Biological Response Modifiers Program, National Cancer Institute, Frederick, MD) (100% of the cells) and MHC-I and MHC-II molecules (100 and 30%, respectively). HLA-A types of this cell line are HLA-A1, A2, and identical the patient's PBL- and EBV-transformed B lymphoblasts.

Hapten Modification. Autologous B lymphoblastoid cells and melanoma cells were modified with DNP by the methods previously described. The DNP modification was confirmed by flow cytometry with a mouse monoclonal anti-DNP antibody (SPE-7; Sigma Chemical Co., St. Louis, MO). After DNP modification, cells were fixed with 70% ethanol on icc for 10 min and then stained with anti-DNP antibody, followed by sheep anti-mouse immunoglobulin antibody conjugated to FITC (Sigma). By this assay, 100% of the cells were shown to be modified with DNP.

Peptide Extraction. Peptides were extracted from MHC molecules of

DNP-Mel, unmodified melanoma cells (MEL), DNP-modified B lymphoblastoid cells (*DNP-LY*), and unmodified B lymphoblastoid cells (*Ly*) by the modified method of Rötzschke *et al.* Science 1990, 249: 283-87. Briefly, 10^9 cells were suspended in 0.1% trifluoroacetic acid (TFA; pH 2.2) and then in 1% TFA, disrupted with a homogenizer and sonicator, and stirred at 4°C for 30 min. Insoluble fractions were removed by centrifugation at 141,000g for 30 min and samples were thereafter lyophilized to remove the organic solvent. The released peptides were then reconstituted in 0.1% TFA and 5% acetonitrile, separated from proteins using a Centricon-10 (Amicon, Bedford, MA) ultrafiltration device (10 kD cutoff), and fractionated by use of reverse-phase HPLC on a Vydac C4 column (150 x 4.6 mm; 5 μm) (The Nest Group, Southborough, MA) on a Hewlett Packard 1050 HPLC system. HPLC solvents consisted of Buffer A (0.1% TFA, 5% acetonitrile, and 95% H_2O) and Buffer B (0.1% TFA, 99.9% acetonitrile). Gradients consisted of the following linear step intervals; 0 to 5 min, 100% A; 5 to 45 min, 25% B (in A); 45 to 55 min, 50% B (in A); and 55 to 60 min, 100% B. Injection volume was 150 μl and flow rate was 1 ml/min. Absorbance was measured at 214 nm. Fractions were collected and dried by lyophilization.

Cytokine Production in Response to Peptides. T cell responses to eluted peptides were measured by IFN-γ production. Each fraction of lyophilized peptides was reconstituted in 200 μl of PBS. EBV-transformed autologous lymphocytes were inactivated by mitomycin C and plated into a 96-well round bottom plate at 2 x 10^4 in 50μl of lymphocyte culture medium. Ten microliters of peptide solution was added into each well, and incubated for 1 hr at 37°C. After this incubation period, T cells were added into each well at 10^5/well in 100 μl of culture medium and incubated for 18 hr, after which supernatants were collected for IFNγ assay. IFNγ production of autologous B lymphoblasts that had not been peptide loaded was regarded as background activity. For batch analysis, 5 μl peptide solution was collected from each of 10 fractions, and 25 μl of the combined peptide fractions,

was added to each well.

IFNγ Assay. The concentration of IFNγ in supernatants was measured by a commercially available ELISA kit (Endogen, Boston, MA; sensitivity, 5 pg/ml). The specific reaction to each peptide fraction was defined as IFN-γ (sample)-IFN-γ (background).

Spectrometric Analysis of DNP-Modified Peptides. DNP modification of the peptides was determined by analytical HPLC on a Vydac C18 column (25 x 4.6 mm; 5 μm). Two HPLC solvents, Buffer A (0.1% TFA, 99.9% H_2O) and Buffer B (0.1% TFA, 99.9% acetonitrile) were used in programmed gradient of 5–25% Buffer B over 40 min (0.5%/min). The fractionated peptides were injected into the analytical HPLC system at flow Vrate of 1 ml/min and absorbance was measured by spectroscopy at 200–380 nm. The eluted peptides were assumed to be DNP-modified if an absorbance peak at 330–360 nm was detected (16).

T Cell Responses to DNP-Modified Autologous Melanoma Peptides. MHC-associated peptides eluted from DNP-Mel were loaded onto B lymphoblastoid cells and tested for their ability to stimulate a T cell line responsive to intact DNP-Mel. T cells predominantly responded to a single peptide fraction 15 with a lower response to the adjacent peptide fraction 14.

Comparison of Peptides Eluted from Melanoma Cells and B Lymphoblasts. The difference in T cell responses to DNP-Mel, unmodified Mel peptides, to DNP-LY and unmodified lymphocyte peptides, was evaluated. A new batch of peptides was prepared by growing 10^9 cells from an autologous melanoma cell line (Mel) and EBV-transformed B lymphoblasts. The cells were either unmodified or modified with DNP. Peptides were eluted from all four cell types. Initially, HPLC fractions were pooled into groups of 10. T cells only responded to the pool containing peptide fractions 11–20 from DNP-Mel. They did not respond to other peptide pools from DNP-LY, lymphocytes, or unmodified MEL.

Next, individual peptide fractions of pools 11–20 from DNP-Mel, unmodified Mel, DNP-LY, and unmodified lymphocytes were added to autologous B

lymphoblasts and tested for IFNγ production by DNP-specific T cells. T cells responded to the DNP-Mel peptide fraction 18 and, to a lesser extent, to peptide fraction 17. The difference between this result and the result obtained in the first DNP-Mel peptide batch 19 most likely to be due to a slight variability in HPLC conditions. Peptide fractions 17 and 18 eluted from DNP-LY were also stimulatory, although the response to fraction 18 from DNP-Mel was much greater than that from *DNP-LY*. No significant response was evoked by any fractions containing unmodified autologous cell peptides. The T cells also proliferated after stimulation with DNP-Mel fractions 17 and 18 (stimulation index, 2.4 and 3.9, respectively) and *DNP-LY* fraction 17 (stimulation index, 2.0) but did not respond to other fractions by proliferation (data not shown).

Demonstration That Stimulatory Peptides Are DNP Modified. To determine whether peptides in the stimulatory fractions were DNP modified, selected fractions were analyzed by spectroscopy at 200–380 nm wavelengths using shallow gradients as described under Materials and Methods. Individual peptide fractions 11 to 20 from DNP-Mel were examined. Peptides in fraction 18 from DNP-Mel eluted at 6.4 to 6.9 min exhibited absorption at 330 nm, indicating that they were DNP modified. In contrast, none of the other nine peptide fractions from DNP-Mel elicited peaks at 330–360 nm. A 330-nm peak was also detectable in peptide fraction 17 from DNP-LY.

The spectroscopy data were confirmed by blocking experiments with anti-DNP antibody. IFNγ production by DNP-specific T cells stimulated by DNP-Mel fraction 18 was almost completely blocked by anti-DNP antibody.

These results demonstrate that immunization with autologous melanoma cells modified with the hapten DNP induces a T cell response against hapten-modified, MHC-associated peptides. These findings are consistent with observations that TNP-specific T cell responses are directed to peptides anchored to the MHC molecules, and not to the hapten-modified MHC molecules themselves. TNP modification of intact cells leads to the production of TNP-modified, MHC-

associated peptides. Such TNP-modified, MHC-associated self-peptides form antigenic epitopes for TNP-specific T cells; and TNP is a critical element for the T cell response. Two types of T cell responses to hapten-modified, MHC-associated peptides were observed. The first type is *sequence independent:* TNP-specific T cell clones recognize a variety of TNP-modified peptides provided that TNP-lysine is located at position 4 and that appropriate anchoring side chains for the MHC groove are present. The second type of T cell recognition of hapten-modified cells is *sequence dependent:* a minor fraction of TNP-specific T cell clones recognize only certain sequences of TNP-modified peptides.

Interestingly, these T cell clones also recognize unmodified peptides; *i.e.*, there was associative recognition of *unmodified* peptides by T cell clones generated by immunization with hapten-modified peptides. Without being bound by any theory, this phenomenon might explain the present findings that hapten-modified autologous melanoma cell vaccine induces inflammation in remote metastases and that the inflammatory response includes T cell clones with unique structures that recognize unmodified melanoma cells. Vaccination with hapten-modified autologous melanoma cells induces T cells which react with hapten-modified tumor peptides on the MHC of melanoma cells. A minor fraction of these T cells may associatively recognize unmodified tumor peptides and consequently be attracted to the metastatic sites. This can be evaluated further by clonal analysis of the DNP-specific T cell line and sequence analysis of the stimulatory fraction of DNP-Mel.

The described DNP-specific T cell line responded predominantly to a single fraction of peptides extracted from the DNP-modified autologous melanoma cell line. The spectrometric analyses of the stimulatory DNP-Mel peptides fraction 18 suggest that multiple peptides in this fraction were modified with DNP.

EXAMPLE 5: Identification of New Melanoma Antigens

Immunogenic melanoma antigens isolated and identified as described in Example 4 are used in a method for screening for new melanoma antigens. The

HPLC fractions to which T cells responded as described in the above Example, and that contain MHC-bound melanoma peptides are saved and analyzed for peptide structure. The peptides are then sequenced to determine their primary structure using methods generally known in the art. For example, the entire peptide pool may be sequenced as described in Burrows *et al.*, *J. Neurosci. Res.* 49:107-116 (1997) and Gavin *et al.*, *Eur. J. Immunol.*, 24:2124-2133, both of which are incorporated herein by reference. Alternatively, individual peptide may be isolated by further fractination of isolated peptide pools using techniques known in the art and then sequenced. *See e.g.* techniques disclosed in U.S. Patent Nos. 5,747,269; 5,487,982; 5,827,516 and 5,820,862. Once the amino acid structure of melanoma peptides is known, synthetic peptides having at least one amino acid variation in this structure are synthesized and tested for their ability to stimulate T cells using the same assay which was used to identify HPLC fractions containing melanoma peptide.

EXAMPLE 6: **Protein From Which Haptenized Peptide Is Derived**

The identification of naturally processed tumor peptides that can stimulate a tumor-specific, CTL response is crucial to the development of a vaccine-based, immunotherapeutic approach to cancer treatment. The tumor-specific CTLs are HLA restricted. Immunoaffinity chromatography is used to isolate the HLA molecules from the tumor cell line, and peptides are eluted with acid from the HLA molecules, and subjected to up to three rounds of separation by reversed phase HPLC. To determine which fractions contains the peptide recognized by tumor-specific CTLs, an aliquot of each HPLC fraction is added to an autologous, B-lymphoblastoid cell line, after which the cells are tested as targets for tumor-specific CTLs. After the final round of reverse-phase-HPLC, mass spectrometry is used to sequence individual peptide candidates, and a peptide with a defined mass/change (m/z) is identified as the active peptide. Collision-activated dissociation allows identification of the peptide sequence.

The peptide sequence so obtained is likely to be identical to a known

protein. PCR can be used to amplify the gene encoding this protein, which can be cloned, expressed, and tested as a T cell antigen and immunogen. These results demonstrate the usefulness of this approach for identifying tumor-specific antigens that are targets of a CTL response.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, and publications are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties.

WHAT IS CLAIMED

1. A method of screening for a tumor antigen that stimulates a specific T cell response directed against a human tumor, which method comprises evaluating a response of a T cell having the property of infiltrating the tumor contacted with a sample, wherein the sample contains a haptenized peptide antigen isolated from an MHC molecule under conditions such that the haptenized peptide antigen is presented to the T cell.

2. The method according to claim 1, wherein the response is selected from the group consisting of:

(i) production of a cytokine by said T cell;

(ii) cytotoxicity of said T cell against said tumor; and

(iii) proliferation of said T cell.

3. The method according to claim 2, wherein the response is release of a cytokine.

4. The method according to claim 1, wherein the peptide is isolated by hapten-specific affinity chromatography.

5. A method for identifying a tumor antigen, which method comprises determining the structure of a haptenized peptide screened according to the method of claim 1.

6. The method according to claim 5, wherein determining the structure of the haptenized peptide comprises mass spectrometry analysis.

7. The method according to claim 6, further comprising peptide mass fingerprint analysis, whereby the protein antigen is identified.

8. A method of screening for a tumor antigen that (i) elicits tumor profiltration of T lymphocytes in a human patient or (ii) causes an inflammatory immune response against the tumor, which method comprises:

(i) isolating a haptenized peptide presented by MHC molecules from a haptenized tumor cell, wherein the peptide stimulates tumor-specific T cells *in vitro*;

(ii) determining the structure of the peptide isolated in step (i);

(iii) preparing a peptide antigen having the structure of the peptide determined in step (ii) except that at least one of the amino acids in said peptide antigen is altered; and

(iv) evaluating the ability of the peptide antigen of step (iii) to stimulate T cells.

9. The method according to claim 8, wherein T cell stimulation is evaluated by a method selected from the group consisting of:

(i) production of a cytokine by said T cell;

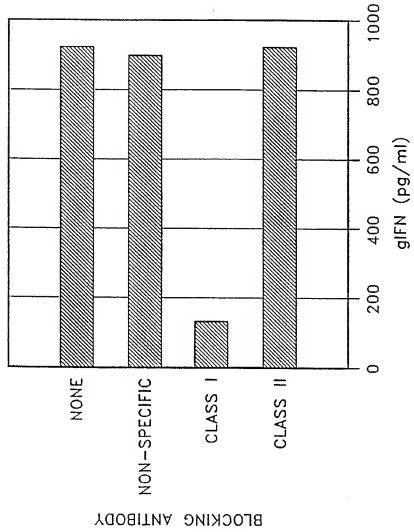
(ii) cytotoxicity of said T cell against said tumor; and

(iii) proliferation of said T cell.

10. The method according to claim 9, wherein the response is release of a cytokine.

11. The method according to claim 8, wherein the peptide is isolated by hapten-specific affinity chromatography.

12. The method according to claim 8, wherein the structure of the peptide is determined by mass spectrometric analysis.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CLASS II
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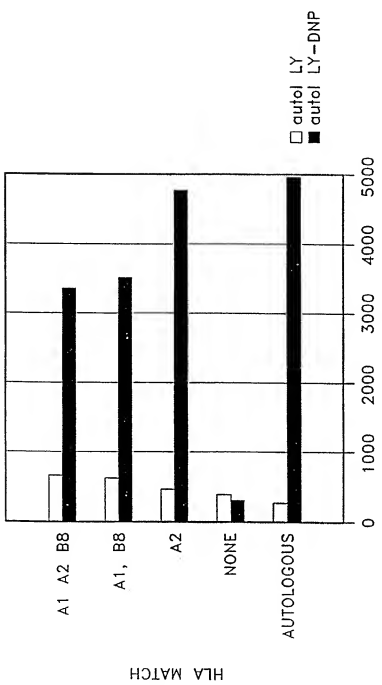
400
600
800
1000
gIFN (pg/ml)

FIG. 1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■ autol LY−DNP
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3000
4000
5000
CPM
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A2
NONE
AUTOLOGOUS
HLA MATCH

FIG. 2

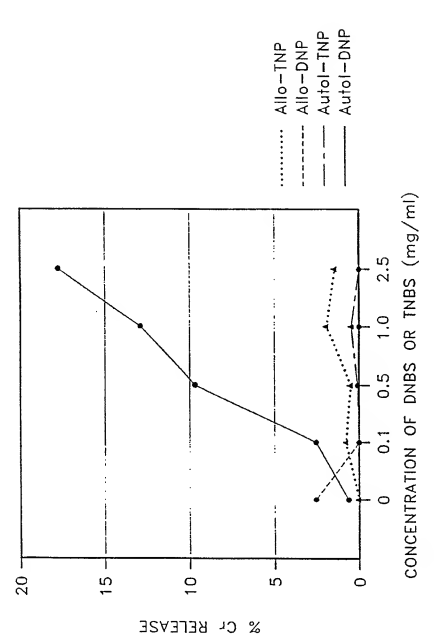
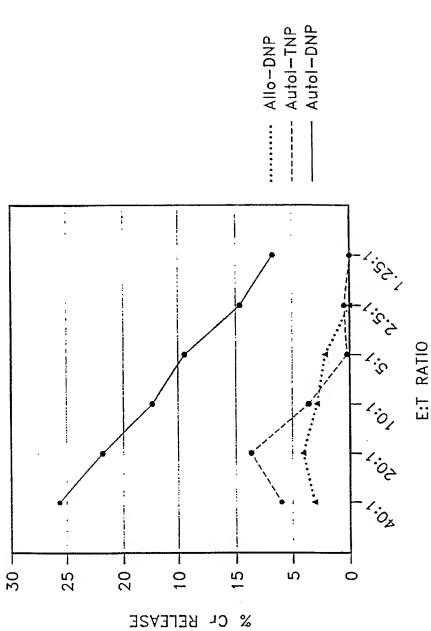

Allo-TNP
Allo-DNP
Autol-TNP
Autol-DNP
% Cr RELEASE
20
15
10
5
0
0
0.1
0.5
1.0
2.5
CONCENTRATION OF DNBS OR TNBS (mg/ml)

FIG. 3A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Autol–TNP
Autol–DNP
% Cr RELEASE
30
25
20
15
10
5
0
40:1
20:1
10:1
5:1
2.5:1
1.25:1
E:T RATIO

FIG. 3B

T CELL STIMULATION BY PEPTIDES
FROM DNP-MODIFIED MELANOMA CELLS

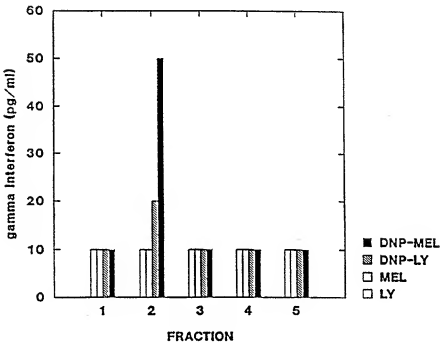

FIG. 4

INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/US 99/28010

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/574 G01N33/68 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 40173 A (THOMAS JEFFERSON UNIVERSITY) 19 December 1996 (1996-12-19) cited in the application page 7, line 1 - line 14; claim 42 page 33, line 25 -page 34, line 12 --- -/--	1-12

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search	Date of mailing of the international search report
24 March 2000	07/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 99/28010

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	MEDLINE, Washington DC USA; abstract no. 95007637, abstract XP002134004 cited in the application & D. BERD ET AL.: "Activation markers on T cells infiltrating melanoma metastases after therapy with dinitrophenyl-conjugated vaccine" CANCER IMMUNOLOGY & IMMUNOTHERAPY, vol. 39, no. 3, 1994, pages 141-147, Philadelphia PA USA	1-12

2

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